

Presenilin-Dependent ErbB4 Nuclear Signaling Regulates the Timing of Astrogenesis in the Developing Brain

S. Pablo Sardi,¹ Joshua Murtie,¹ Samir Koirala,¹ Brooke A. Patten,¹ and Gabriel Corfas^{1,*}

¹Neurobiology Program and Department of Neurology, Children's Hospital and Harvard Medical School, 300 Longwood Avenue, Boston, MA, 02115, USA

*Contact: gabriel.corfas@childrens.harvard.edu

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SUMMARY

Embryonic multipotent neural precursors are exposed to extracellular signals instructing them to adopt different fates, neuronal or glial. However, the mechanisms by which precursors integrate these signals to make timely fate choices remained undefined. Here we show that direct nuclear signaling by a receptor tyrosine kinase inhibits the responses of precursors to astrocyte differentiation factors while maintaining their neurogenic potential. Upon neuregulin-induced activation and presenilin-dependent cleavage of ErbB4, the receptor's intracellular domain forms a complex with TAB2 and the corepressor N-CoR. This complex undergoes nuclear translocation and binds promoters of astrocytic genes, repressing their expression. Consistent with this observation, astrogenesis occurs precociously in *ErbB4* knockout mice. Our studies define how presenilin-dependent nuclear signaling by a receptor tyrosine kinase directly regulates gene transcription and cell fate. This pathway could be of importance for neural stem cell biology and for understanding the pathogenesis of Alzheimer's disease.

INTRODUCTION

Regulation of gene transcription by receptor tyrosine kinases (RTKs) plays essential roles in many cellular processes. Until recently, these receptors were believed to regulate transcription only via the activation of a multilayered network of kinase cascades that phosphorylate transcription factors, altering their activity (Schlessinger, 2000). However, recent studies on ErbB4, a member of the EGF receptor family, suggest that some RTKs may signal directly to the cell nucleus via a novel signaling mechanism that does not depend on kinase cascades but rather requires receptor phosphorylation, intramembrane

proteolysis, and nuclear translocation of the RTK intracellular domain (Lee et al., 2002; Ni et al., 2001).

Neuregulin 1 (NRG1) stimulation of ErbB4 promotes receptor ectodomain cleavage by the metalloprotease TNF- α -converting enzyme (TACE) (Rio et al., 2000). The cell-associated fragment containing the transmembrane and cytoplasmic domains is subsequently cleaved by presenilin/ γ -secretase, releasing its intracellular domain (E4ICD) into the cytosol. E4ICD can then translocate to the nucleus (Ni et al., 2001). The potential biological significance of the proteolytic cleavage of ErbB4 is underscored by two facts. First, ErbB4 appears to be the only member of the ErbB family to be processed by proteases in this fashion and to harbor a functional nuclear localization signal in its intracellular domain (Carpenter, 2003). Second, alternative splicing of the *ErbB4* gene generates two isoforms that differ in their extracellular juxtamembrane (JM) regions, their sensitivity to proteolytic cleavage (Elenius et al., 1997), and therefore their ability to signal through regulated intramembrane proteolysis. Whereas ErbB4 JMa, the originally described isoform of ErbB4 (Plowman et al., 1993), is sensitive to cleavage by TACE and presenilin, the ErbB4 JMb isoform is not. Interestingly, these isoforms have different expression patterns, indicating that proteolytic cleavage of ErbB4, and therefore its nuclear signaling, is specific to certain tissues, in particular the brain, where ErbB4 JMa is highly expressed (Elenius et al., 1997). These observations suggested that ErbB4 cleavage and E4ICD nuclear translocation could play important roles in the brain, but its functions and mechanisms of action remained to be identified.

Here we show that NRG1-induced presenilin-dependent ErbB4 nuclear signaling regulates the timing of astrogenesis in the developing brain. Upon activation and presenilin-dependent cleavage of ErbB4, E4ICD forms a complex with the signaling protein TAB2 and the corepressor N-CoR. This complex translocates to the nucleus of undifferentiated neural precursors and inhibits their differentiation into astrocytes by repressing the transcription of glial genes. Consistent with this observation, cortical astrogenesis occurs precociously in *ErbB4* knockout embryos, a phenotype that is rescued by re-expression of human ErbB4 JMa but not by the uncleavable ErbB4 JMb.

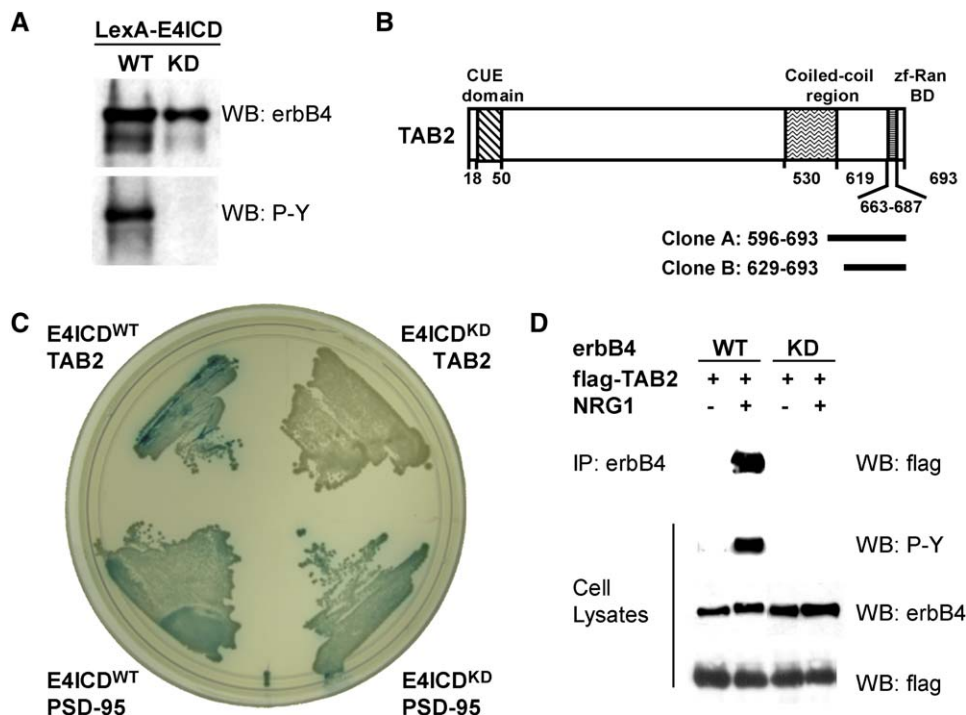


Figure 1. Activated E4ICD Interacts with TAB2 in Yeast and Mammalian Cells

(A) E4ICD becomes phosphorylated in yeast. Yeast expressing wild-type (WT) and kinase-dead (KD) LexA-E4ICD fusion proteins were lysed and immunoblotted with ErbB4 or phosphotyrosine (P-Y) antibodies, showing that both proteins are expressed (top panel) but only the WT is tyrosine phosphorylated (bottom panel).

(B) Schematic diagram of TAB2. Regions of TAB2 included in two clones identified in the screen are indicated. zf-Ran BD: zinc finger Ran-binding domain.

(C) Tyrosine kinase activity of E4ICD is necessary for interaction with TAB2 in yeast. WT or KD LexA-E4ICD was coexpressed in yeast with full-length TAB2 or PDZ domains 1 and 2 of PSD-95 (Huang et al., 2000) as fusion proteins with the B42 activation domain. TAB2 only interacts with phosphorylated E4ICD (blue color), whereas PSD-95 interacts with both baits.

(D) ErbB4/TAB2 interaction in mammalian cells depends on receptor activation. Full-length ErbB4 WT or KD was transfected into HEK293 cells together with full-length FLAG-TAB2. Cells were left untreated or were treated with NRG1, lysed, and immunoprecipitated with ErbB4 antibody followed by immunoblotting with FLAG antibody. Whole-cell lysates were also immunoblotted with ErbB4, phosphotyrosine (P-Y), and FLAG antibodies to characterize the input.

Our studies define how presenilin-dependent nuclear signaling by an RTK directly regulates gene transcription and cell fate in the developing brain.

RESULTS

Identification of Proteins that Interact with Activated E4ICD

Since the E4ICD has no known DNA-binding or transcriptional activation motifs, we hypothesized that ErbB4-binding proteins that are involved in transcriptional regulation should exist and that their interaction with ErbB4 might depend on its activation. To identify such proteins, we used a yeast two-hybrid system that facilitates the isolation of proteins that interact with activated RTKs (Qian et al., 1998). Screening of a cDNA expression library from rat embryonic day 14 (E14) spinal cord and dorsal root ganglia with a bait containing the entire E4ICD in an activated state (Figure 1A) led to isolation of several putative E4ICD-interacting proteins. Among them, two clones

contained cDNAs encoding the C-terminal region of TAB2 (TAK1 binding protein 2; Figure 1B), a protein first identified as an adaptor for TAK1 (transforming growth factor β -activated kinase 1) (Takaesu et al., 2000). Wild-type E4ICD also interacted with full-length TAB2 in yeast, and this association was abolished when the tyrosine kinase activity of E4ICD was eliminated by a mutation in the ATP-binding site (E4ICD^{KD}) (Figure 1C). Thus, binding of TAB2 to ErbB4 in yeast appears to occur only when the receptor is activated.

We then tested whether TAB2 also binds to ErbB4 in mammalian cells and whether this interaction depends on receptor activation by NRG1, an ErbB4 ligand. Cells were cotransfected with FLAG-TAB2 and full-length ErbB4 expression constructs and then subjected to immunoprecipitation with ErbB4 antibodies. TAB2 coprecipitated with ErbB4 only after NRG1 treatment (Figure 1D). In contrast, full-length kinase-dead ErbB4 failed to interact with TAB2 even in the presence of NRG1, indicating that ligand binding is not sufficient to induce TAB2/ErbB4

association (Figure 1D). These results show that ErbB4 and TAB2 interact in mammalian cells and that this association requires receptor activation.

Presenilin-Dependent Cleavage of ErbB4 Promotes TAB2 Nuclear Translocation

The dependence of ErbB4/TAB2 interaction on NRG1 suggested that ErbB4 activation could alter TAB2, particularly its state of phosphorylation or its cellular localization. Phosphotyrosine western blot and ^{32}P incorporation assays in cells expressing ErbB4 did not demonstrate induction of TAB2 phosphorylation by NRG1 (data not shown). Furthermore, no shift of the TAB2 band was observed on western blots in response to NRG1 (Figure 1D). Thus, it appears that ErbB4 activation does not induce TAB2 phosphorylation. In contrast, NRG1-ErbB4 signaling had dramatic effects on the cellular distribution of TAB2. In quiescent NIH 3T3 cells, TAB2 was excluded from the nuclei independently of whether the cells expressed ErbB4 or not. Notably, upon stimulation with NRG1, TAB2 translocated to the nucleus in cells expressing ErbB4 (Figure 2A).

In the experiments described above, we used ErbB4 JMa, the splice variant that undergoes proteolytic cleavage after activation (Elenius et al., 1997). To determine whether NRG1-induced TAB2 nuclear translocation depends on ErbB4 cleavage, we tested whether TAB2 shuttling also occurs in cells that express the naturally occurring TACE-resistant isoform ErbB4 JMb. In contrast to ErbB4 JMa, NRG1 did not induce TAB2 nuclear translocation in cells expressing ErbB4 JMb (Figure 2B), even though this uncleavable isoform interacted with TAB2 upon NRG1 stimulation (data not shown). Furthermore, the NRG1-induced nuclear translocation of TAB2 in ErbB4 JMa-expressing cells was eliminated by the presenilin inhibitor DAPT (Dovey et al., 2001) (Figure 2B). Moreover, ErbB4 JMa^{KD} failed to induce TAB2 nuclear shuttling even in the presence of NRG1 (Figure 2B). These results show that NRG1-induced activation of ErbB4 JMa and the ensuing sequential proteolytic cleavage by TACE and presenilin are important for TAB2 nuclear translocation.

The ErbB4-dependent TAB2 nuclear translocation raised the possibility that TAB2 could be mediating potential effects of E4ICD on gene expression. We tested this using a GAL4/TAB2 fusion protein and a *UAS-TK-luciferase* reporter. The basal activity of this reporter in unstimulated cells is moderate, but it can be either increased or decreased by transcriptional activators or repressors (Chen and Evans, 1995). Treating cells expressing ErbB4 JMa and GAL4/TAB2 with NRG1 significantly reduced *UAS-TK*-directed luciferase activity (Figure 2C). In contrast, NRG1 had no effects on *UAS-TK*-directed luciferase activity when cells expressed ErbB4 JMb or when the ErbB4 JMa-expressing cells were exposed to DAPT (Figure 2C). These findings provided further evidence that NRG1-mediated ErbB4 activation and cleavage induces nuclear translocation of TAB2 and raised the possibility that an E4ICD/TAB2 complex represses transcription.

Given that TAB2 can form a complex with the transcriptional corepressor N-CoR (Baek et al., 2002), we tested whether N-CoR also interacts with ErbB4. Cells were transfected with full-length ErbB4 or ErbB4^{KD}, treated with NRG1, and immunoprecipitated with ErbB4 antibodies. As with TAB2, only wild-type ErbB4 coimmunoprecipitated with endogenous N-CoR, and this occurred only when the cells were stimulated with NRG1 (Figure 2D). No tyrosine phosphorylation of N-CoR was detected when ErbB4 was activated (data not shown). These results show that NRG1 induces the formation of an E4ICD/TAB2/N-CoR complex.

We then probed the roles of TAB2 in the interaction between E4ICD and N-CoR. First, we asked whether ErbB4 could bind to N-CoR in the absence of TAB2 using RNAi. Lack of TAB2 expression abolished the NRG1-dependent E4ICD/N-CoR association, suggesting that TAB2 forms the bridge between E4ICD and N-CoR (Figure 2E). We also used truncated versions of TAB2 to define the specific domains within this protein responsible for its ability to link E4ICD to N-CoR. As expected from the portion of TAB2 that binds to E4ICD in the yeast two-hybrid assay (Figure 1B), a truncated TAB2 lacking its C-terminal end (TAB2 Δ C) failed to coprecipitate with activated ErbB4 (Figure 2F), indicating that the C-terminal 64 amino acids of TAB2 are not only sufficient but also necessary for ErbB4/TAB2 interaction. In contrast, TAB2 binds constitutively to N-CoR (Figure 2G). Furthermore, N-CoR physically interacts with TAB2 Δ C but not with TAB2 Δ N, the C-terminal E4ICD-interaction domain of TAB2 (Figure 2G). These results indicate that TAB2 is a bifunctional molecule with nonoverlapping binding sites for ErbB4 and N-CoR and that it provides the critical link for the formation of the ErbB4/TAB2/N-CoR complex.

ErbB4 Nuclear Signaling Affects Neural Precursor Differentiation In Vitro

Neuronal precursors (NPs) express ErbB4 receptors (Fox and Kornblum, 2005), but the functional relevance of ErbB4 signaling in this population remained undefined. We hypothesized that an E4ICD/TAB2/N-CoR complex could be of biological significance for NPs in the central nervous system. We first characterized the expression of ErbB receptors in primary NP cultures obtained from E14.5 rat cortices. These cells express only the ErbB4 JMa isoform (see Figure S1A in the Supplemental Data available with this article online), which is readily activated by NRG1 (Figure 3A), leading to its cleavage and release of the 80 kDa E4ICD (Figure 3B). NPs also express ErbB2, but not ErbB3 (Figures S1B and S1C). Since ErbB2 does not bind to NRG1, these results indicate that any response of NPs to NRG1 would require ErbB4, acting as either a homodimer or an ErbB4/2 heterodimer.

To test whether ErbB4 endogenous to NPs interacts with TAB2 and N-CoR, we performed immunoprecipitation assays and found that NRG1 induces ErbB4 JMa association with both endogenous TAB2 and N-CoR (Figure 3C). In addition, immunostaining showed that NRG1

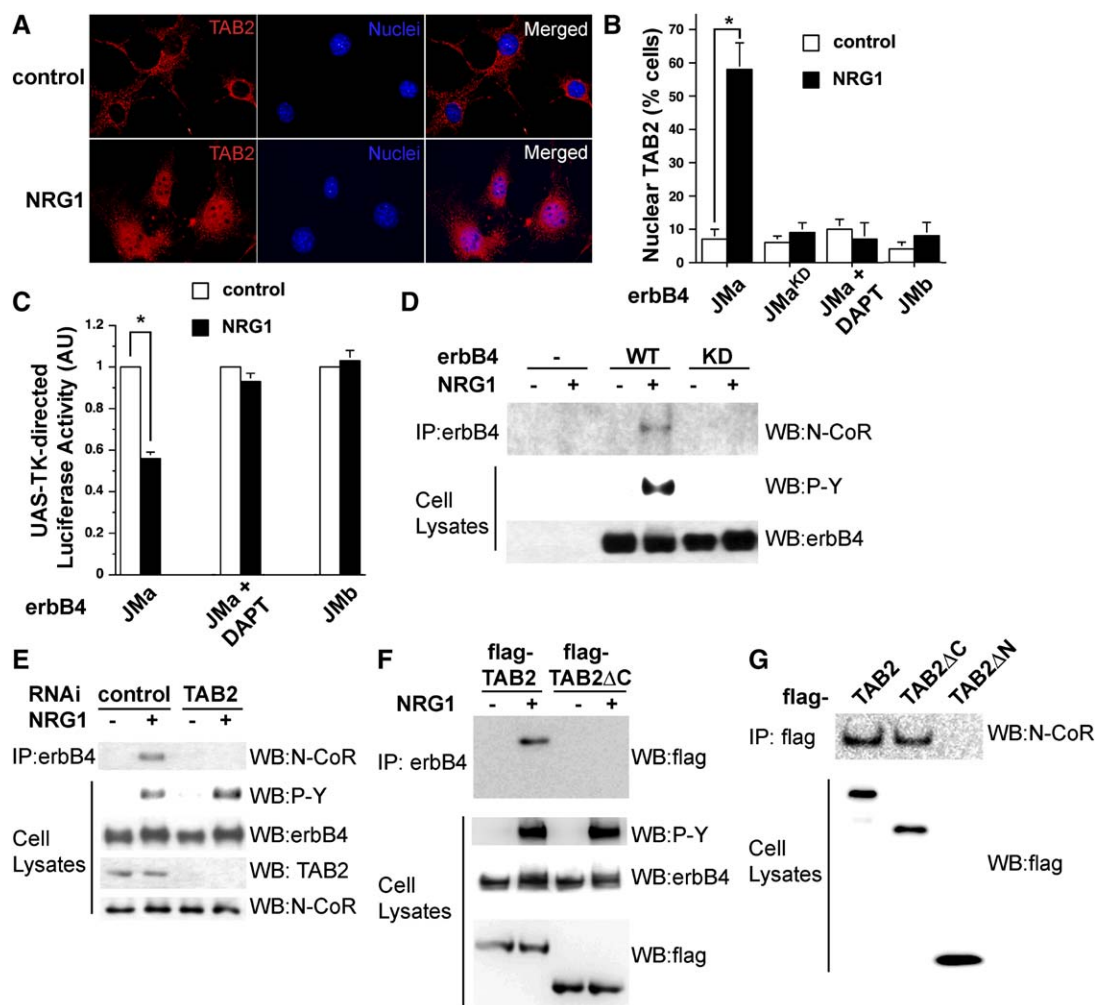


Figure 2. Activated ErbB4 Induces TAB2 Nuclear Translocation and Interacts with N-CoR

(A) Activation of presenilin-sensitive isoform ErbB4 JMa induces nuclear translocation of TAB2. Whereas TAB2 immunoreactivity is excluded from the nucleus in unstimulated cells expressing ErbB4 JMa, treatment with NRG1 for 2 hr results in TAB2 nuclear localization.

(B) NRG1-induced TAB2 nuclear translocation depends on ErbB4 activation and cleavage. Whereas treatment with NRG1 for 2 hr induces a dramatic increase in TAB2 nuclear localization in cells expressing ErbB4 JMa, this translocation does not occur if cells are exposed to the presenilin inhibitor DAPT 30 min before NRG1 stimulation or if cells express the cleavage-resistant isoform ErbB4 JMb or ErbB4 JMa^{KD} (*p < 0.01). In this and all other figures, error bars represent the standard error of the mean (SEM).

(C) ErbB4 JMa-induced TAB2 nuclear translocation represses transcription. Cells expressing ErbB4 JMa or JMb were cotransfected with a GAL4-TAB2 fusion plasmid, a UAS-TK-luciferase reporter, and a TK-renilla reporter. Twenty-four hours later, cells were treated with DAPT or vehicle, followed by stimulation with NRG1. Cells were lysed 24 hr later, and luciferase activity was measured (*p < 0.05).

(D) Activated ErbB4 interacts with N-CoR. Cells were transfected with WT or KD ErbB4, treated with NRG1 for 30 min, lysed, and subjected to immunoprecipitation with an ErbB4 antibody followed by immunoblotting with N-CoR. Whole-cell lysates were immunoblotted with ErbB4 and phosphotyrosine antibodies.

(E) TAB2 knockdown blocks ErbB4/N-CoR interaction. Cells transduced with control or TAB2 RNAi-expressing lentiviruses were treated and processed as in (D).

(F) TAB2 C terminus is necessary for ErbB4/TAB2 interaction. ErbB4 was transfected together with FLAG-TAB2 (full-length or Δ C truncated protein). Cells were treated and processed as in (D).

(G) TAB2 N terminus interacts with N-CoR. Cells were transfected with FLAG-TAB2 (full-length or Δ C or Δ N truncated proteins). After lysis and immunoprecipitation with FLAG antibody, samples were immunoblotted with N-CoR antibody. Whole-cell lysates were also immunoblotted with FLAG antibody to characterize the input. FLAG-TAB2, 88 kDa; FLAG-TAB2 Δ C, 80 kDa; FLAG-TAB2 Δ N, 18 kDa.

promotes nuclear translocation of E4ICD, TAB2, and N-CoR in virtually all NPs and that this depends on presenilin activity (Figures 3D and 3E). Furthermore, lentivirus-mediated

RNAi knockdown of TAB2 (Figure S2A) abolished the NRG1-induced nuclear translocation of N-CoR, whereas E4ICD nuclear translocation was not affected (Figure 3F).

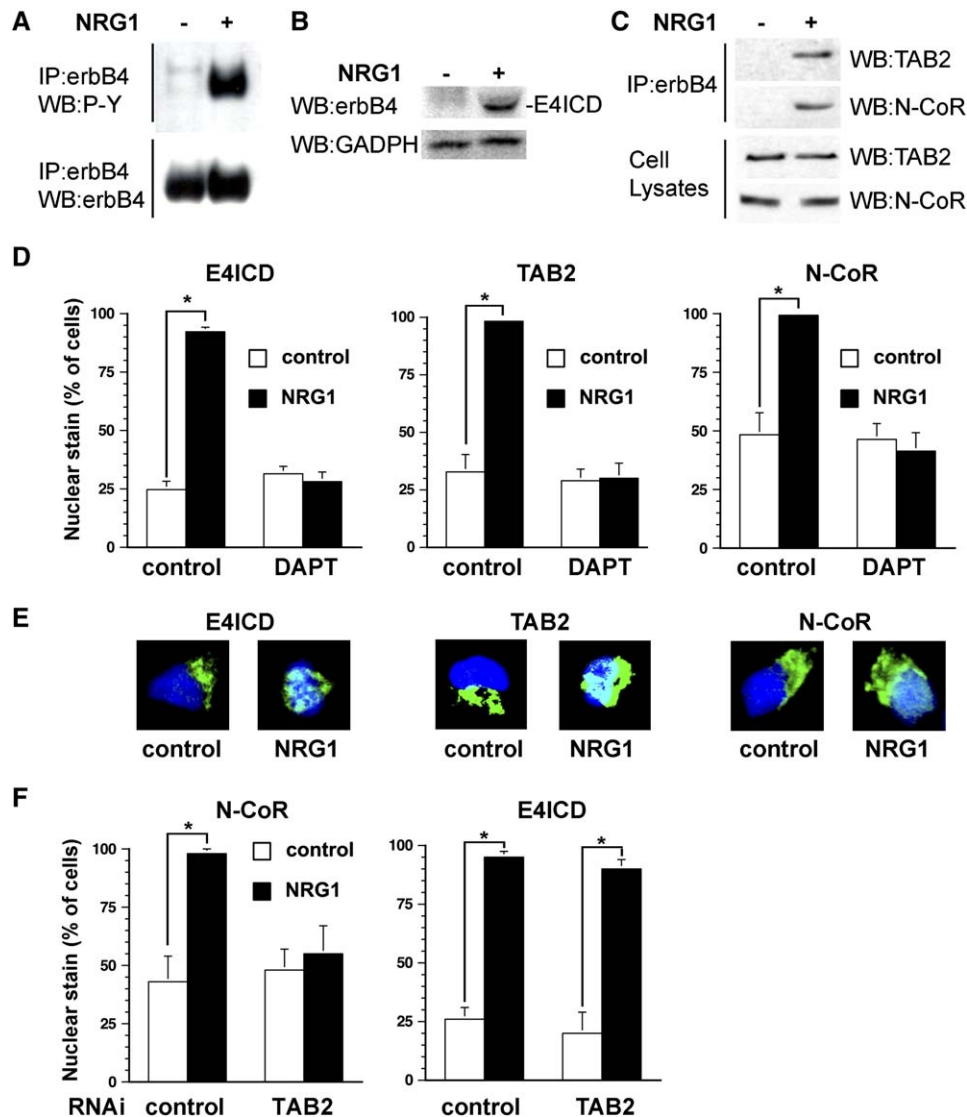


Figure 3. Neural Precursors Express ErbB4 JMa, which, When Activated, Promotes the Formation and Nuclear Translocation of an E4ICD/TAB2/N-CoR Complex

(A) NRG1 treatment (5 min) induces ErbB4 phosphorylation in neural precursors (NPs).

(B) NRG1 stimulation promotes E4ICD release from NPs. Cytoplasmic fractions of NPs either left untreated or stimulated with NRG1 were subjected to western blot with ErbB4 antibodies. GADPH was used to control for loading.

(C) NRG1 induces ErbB4 JMa interactions with endogenous TAB2 and N-CoR in NPs. Cells were either left untreated or stimulated with NRG1 (30 min), lysed, immunoprecipitated with ErbB4 antibodies, and immunoblotted with N-CoR or TAB2 antibodies. Whole-cell lysates were blotted with the same antibodies to show the input.

(D) NRG1 treatment (2 hr) stimulates nuclear translocation of endogenous E4ICD, TAB2, and N-CoR in NPs in a presenilin-dependent fashion (* $p < 0.01$).

(E) Representative images of untreated NPs (control) or NPs stimulated with NRG1 stained with ErbB4, TAB2, or N-CoR (green). Nuclei are labeled with Hoechst (blue).

(F) RNAi knockdown of *TAB2* inhibits NRG1-dependent N-CoR nuclear translocation, while E4ICD nuclear shuttling is not affected. NPs were transduced with control or *TAB2* RNAi-expressing lentiviruses. Three days later, cells were treated and analyzed as in (D). Identical results were obtained with two different RNAi constructs against *TAB2* (* $p < 0.01$).

These results indicate that E4ICD might be responsible for nuclear shuttling of the E4ICD/TAB2/N-CoR complex in NPs.

We then tested whether NRG1 stimulation of ErbB4 JMa nuclear signaling could regulate aspects of NP biol-

ogy. Multipotent NPs can be isolated from embryonic brains and maintained in culture in a proliferative undifferentiated state or can be induced to adopt astrocytic or neuronal fates by extracellular signaling molecules. These molecules include ciliary neurotrophic factor (CNTF) and

platelet-derived growth factor (PDGF), which induce astrocytic or neuronal differentiation, respectively (Johe et al., 1996). NRG1 stimulation did not induce the acquisition of either neuronal or astrocytic fates, as defined by expression of the cell-type-specific markers glial fibrillary acid protein (GFAP) and β -tubulin III (Figures 4A and 4B). Similarly, NRG1 treatment did not modify NP survival or proliferation (data not shown). Surprisingly, NRG1 antagonized the effects of CNTF on astrogenesis (Figure 4A) without altering the ability of PDGF to induce NPs to adopt a neuronal fate (Figure 4B). Furthermore, the presenilin inhibitor DAPT blocked the effect of NRG1 on CNTF-induced astrogenesis (Figure 4A), indicating that cleavage of the ErbB4 JMa receptor is required for this inhibition of astrogenesis. These results suggest that activation and cleavage of ErbB4 JMa after NRG1 stimulation might contribute to maintenance of the NP pool in a neurogenic state by preventing their differentiation into astrocytes.

To determine whether ErbB4 is the NRG1 receptor implicated in this differentiation effect, we eliminated ErbB4 expression in NPs using lentivirus-mediated RNAi knockdown (Figure S2B). Infection with control lentivirus did not alter the number of GFAP-positive astrocytes found in untreated, NRG1-treated, and/or CNTF-treated cultures (Figure 4C). In contrast, knockdown of *ErbB4* completely abolished the ability of NRG1 to antagonize the CNTF-induced astrogenesis (Figure 4C), indicating that this receptor is essential for the NRG1-mediated effect. Similar experiments carried out with *TAB2* RNAi produced identical results (Figure 4D), showing that *TAB2* is required for the inhibition of the NRG1-dependent inhibition of astrocyte differentiation.

E4ICD Nuclear Signaling Inhibits Astrogenesis through Transcriptional Repression of Astrocytic Genes

CNTF induction of NP differentiation into astrocytes involves transcriptional activation of the *GFAP* promoter (Bonni et al., 1997). Consistent with the ability of ErbB4 signaling to antagonize the effects of CNTF on astrocyte differentiation, NRG1 had no effect on *GFAP* promoter basal activity in a luciferase reporter assay in NPs, but it significantly reduced the effects of CNTF on this promoter's activity (Figure 5A). This antagonistic effect of NRG1 was blocked by the presenilin inhibitor DAPT (Figure 5A), expression of dominant-negative forms of presenilin (Figure S3), and knockdown of *ErbB4* (Figure 5B). Importantly, NRG1 had similar effects on *S100 β* , another astrocyte protein. As with the *GFAP* promoter, NRG1 had no effect on the basal activity of a *S100 β* -luciferase reporter in NPs, it significantly reduced its activation by CNTF, and this antagonistic effect of NRG1 was blocked by the presenilin inhibitor DAPT (data not shown). These results indicate that NRG1 inhibits astrocyte differentiation through a transcriptional mechanism that depends on presenilin-mediated cleavage of ErbB4.

The requirement of ErbB4 cleavage in the inhibition of the CNTF effects was further investigated by testing

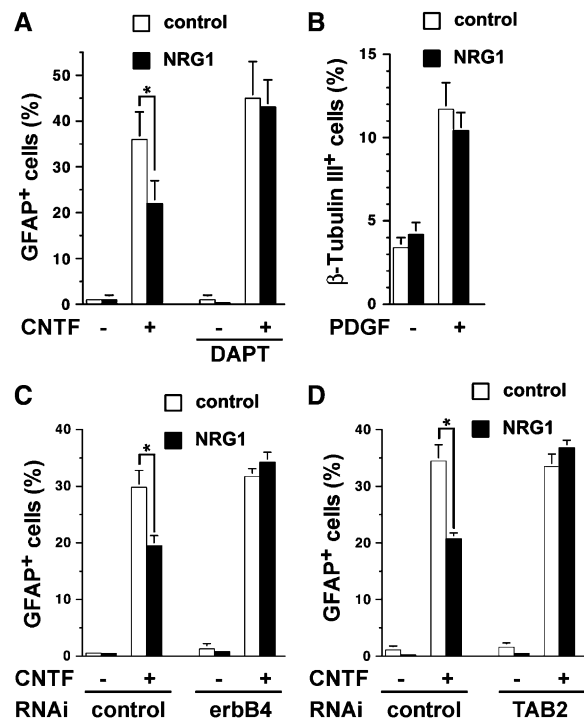


Figure 4. NRG1-ErbB4 Signaling Inhibits Astrogenesis in a Presenilin-Dependent Fashion

(A) NRG1 blocks CNTF-induced differentiation of NPs into astrocytes in a presenilin-dependent manner. CNTF treatment (2 days) induced NPs to differentiate into GFAP-expressing astrocytes. NRG1 treatment did not change the number of GFAP⁺ cells but significantly reduced the CNTF-mediated astrogenesis if applied 3 hr prior to CNTF. The NRG1 inhibition of CNTF-induced astrogenesis was blocked by addition of DAPT 30 min prior to the other treatments (* $p < 0.05$).

(B) NRG1 has no effect on PDGF-induced neurogenesis. PDGF treatment (4 days) increased the number of cells expressing the neuronal marker β -tubulin III. Addition of NRG1 alone or before PDGF did not alter the number of β -tubulin III⁺ cells.

(C and D) *ErbB4* or *TAB2* knockdown blocks NRG1 inhibition of astrogenesis. NPs were transduced with control or *TAB2* or *ErbB4* RNAi-expressing lentiviruses. Three days later, cells were treated with CNTF and/or NRG1 and analyzed as in (A). Similar results were obtained with two different RNAi constructs against *ErbB4* or *TAB2* (* $p < 0.05$).

whether ErbB4 variants with diverse sensitivities to proteases could rescue the effects of NRG1 after RNAi knockdown of endogenous *ErbB4*. ErbB4 receptors endogenous to NPs were knocked down with *ErbB4* RNAi, and 3 days later, cells were transfected with cDNAs encoding one of the human ErbB4 juxtamembrane isoforms (HER4 JMa or JMb) or a mutant HER4 insensitive to presenilin cleavage (HER4 JMa V673I), all of which are resistant to the RNAi. Cells were cotransfected with the *GFAP*-luciferase and *TK*-renilla reporters. As shown in Figure 5B, knockdown of endogenous *ErbB4* abolished the antagonistic effect of NRG1 on *GFAP* promoter activity. Notably, the antagonizing effect of NRG1 on CNTF-mediated *GFAP* activation was rescued only by expression of HER4 JMa, but not by the TACE-resistant HER4 JMb or the

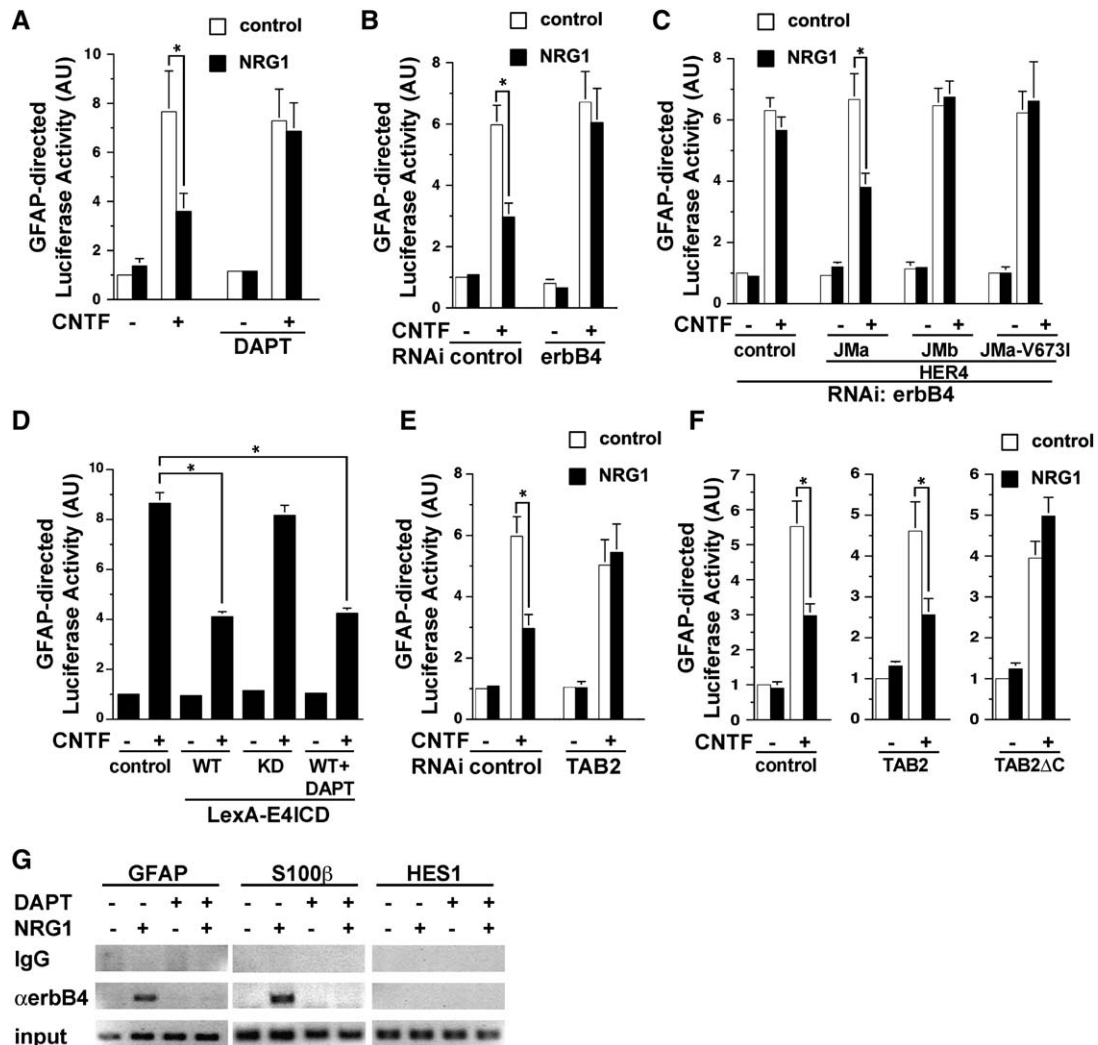


Figure 5. NRG1-ErbB4 Nuclear Signaling Inhibits GFAP Promoter Activation and Induces E4ICD Recruitment to this Promoter

(A) NRG1 blocks CNTF-induced GFAP promoter activation in a presenilin-dependent manner. NPs were cotransfected with GFAP-luciferase and TK-renilla reporters. The next day, cells were treated with NRG1, CNTF, and/or DAPT. DAPT was added 30 min prior to NRG1. NRG1 was added 3 hr prior to CNTF. Six hours after CNTF addition, NPs were lysed and luciferase activity was measured (* $p < 0.05$).

(B) NRG1-mediated inhibition of CNTF-induced GFAP promoter activation depends on ErbB4. NPs were transduced with control or ErbB4 RNAi-expressing lentiviruses. After 3 days, cells were transfected, treated with NRG1 and/or CNTF, and analyzed as in (A). Similar results were obtained with two different RNAi constructs against ErbB4 (* $p < 0.05$).

(C) Only the cleavable isoform HER4 JMa rescues the ErbB4 knockdown phenotype. ErbB4 endogenous to NPs was knocked down by RNAi. After 3 days, the human ErbB4 juxtamembrane isoforms (HER4 JMa or JMb) or the presenilin-resistant HER4 JMa V673I, which are resistant to the RNAi, were transfected along with the reporters. Cells were then treated and processed as in (A) (* $p < 0.05$).

(D) Expression of activated E4ICD is sufficient to inhibit CNTF-induced GFAP promoter activation. NPs were cotransfected with empty vector, WT, or KD LexA-E4ICD plasmids along with the reporters. The next day, cells were treated with CNTF and processed as in (A). Only wild-type E4ICD blocked CNTF induction of the GFAP promoter; this blockade was presenilin independent (* $p < 0.05$).

(E) NRG1-mediated inhibition of CNTF-induced GFAP promoter activation depends on TAB2. NPs were transduced with control or TAB2 RNAi-expressing lentiviruses. After 3 days, cells were transfected, treated, and analyzed as in (A). Similar results were obtained with two different RNAi constructs against TAB2 (* $p < 0.05$).

(F) NRG1-mediated inhibition of CNTF-induced GFAP promoter activation requires the TAB2 C terminus. TAB2 (full-length or ΔC truncated) was transfected along with the reporters. Cells were then treated and processed as in (A) (* $p < 0.05$).

(G) NRG1 induces E4ICD binding to the GFAP and S100β promoters in a presenilin-dependent manner. NPs were incubated for 30 min with vehicle or DAPT before NRG1 treatment. After 6 hr, ChIP assays were performed with antibodies to ErbB4 or control IgG. PCR primers specific for GFAP or S100β promoters were used. Primers against the first exon of HES1 serve as a negative control.

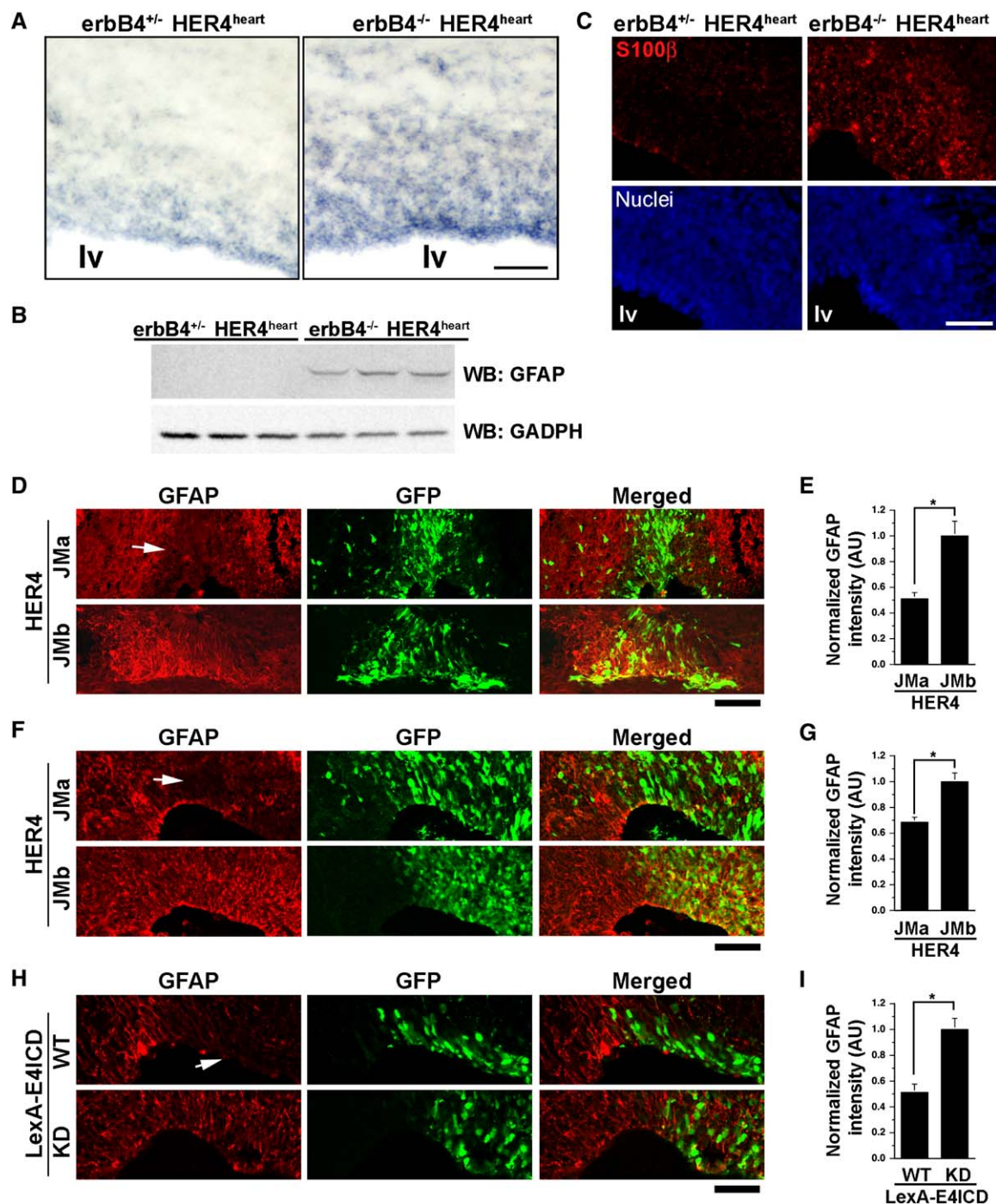


Figure 6. Precocious Astrogenesis in *ErbB4* Knockout Developing Cortex

(A) Increased GFAP mRNA expression in E17.5 *ErbB4*^{-/-} *HER4*^{heart} mouse cortex. In situ hybridization on coronal sections showing GFAP mRNA expression in *ErbB4*^{+/-} and *ErbB4*^{-/-} *HER4*^{heart} littermates. No signal was generated using a sense probe. lv = lateral ventricle. Bar = 50 μ m.

(B) Increased GFAP protein expression in brains of E17.5 *ErbB4*^{-/-} *HER4*^{heart} mice. GFAP immunoblot of whole-brain lysates of *ErbB4*^{-/-} *HER4*^{heart} embryos and their *ErbB4*^{+/-} *HER4*^{heart} littermates is shown. Three independent embryos are shown for each genotype. The membrane was stripped and reblotted with GADPH antibody as a loading control.

(C) Increased expression of the astrocytic marker S100 β in E17.5 *ErbB4*^{-/-} *HER4*^{heart} mouse cortex. Coronal sections show S100 β immunostaining and nuclear staining in *ErbB4*^{+/-} and *ErbB4*^{-/-} *HER4*^{heart} littermates. lv = lateral ventricle. Bar = 50 μ m.

(D) Only *HER4* JMa rescues the *ErbB4*^{-/-} phenotype in slice cultures. Day 13.5 *ErbB4*^{-/-} *HER4*^{heart} embryos were electroporated in utero with *HER4* and GFP plasmids and dissected immediately. Forebrain slices were cultured for 4 days in vitro and processed for GFAP immunofluorescence.

presenilin-resistant HER4 JMa V673I (Figure 5C). These results indicate that activation of the canonical signaling by the ErbB4 RTK is not sufficient to exert this action. In order for ErbB4 to block CNTF-mediated GFAP expression, cleavage of this RTK is absolutely necessary.

So far, our results indicated that E4ICD is necessary for the effects of NRG1 on astrogenesis. To determine whether E4ICD activity is sufficient to promote the GFAP transcriptional repression, we used the LexA-E4ICD fusion that, when expressed in mammalian cells, dimerizes, becomes autophosphorylated, and interacts with TAB2 (Figure S4). Expression of LexA-E4ICD in NPs had no effect on the activity of the GFAP promoter, but it antagonized the CNTF-mediated GFAP activation (Figure 5D). This effect of LexA-E4ICD was abolished when E4ICD was rendered kinase dead but was not affected by the presenilin blocker (Figure 5D). These results show that E4ICD nuclear signaling is necessary and sufficient for the inhibition of astrogenesis by NRG1.

To further investigate the roles of TAB2 in these processes, we used RNAi to knock down its expression. Similar to *ErbB4*, knockdown of *TAB2* eliminated the antagonistic effect of NRG1 on GFAP expression (Figure 5E). Interestingly, overexpression of TAB2 Δ C, the truncated TAB2 that binds to N-CoR but not to ErbB4, also blocked the antagonistic effect of NRG1 on CNTF-mediated transcriptional activation of GFAP, whereas full-length TAB2 did not (Figure 5F), indicating that TAB2 Δ C acts as a dominant-negative molecule by preventing formation of the complex. These results indicate that repression of the GFAP promoter by ErbB4 nuclear signaling requires the presence of TAB2, which brings together E4ICD and N-CoR.

The results described above suggested that E4ICD could be part of the transcriptional repressor complex that mediates the NRG1 inhibition of GFAP and *S100* β expression. To test this possibility, we used a chromatin immunoprecipitation (ChIP) assay. Immunoprecipitation with ErbB4 antibodies showed that E4ICD associates with the GFAP and *S100* β promoters in NPs, but only after treatment with NRG1 (Figure 5G). Moreover, these associations were blocked by presenilin inhibition (Figure 5G). The association of E4ICD with the glial promoters was specific since normal rabbit immunoglobulin G (IgG) failed to immunoprecipitate these promoters and ErbB4 anti-

bodies did not precipitate a control promoter, *HES1* (Figure 5G). These results indicate that NRG1 activation of ErbB4 induces the presenilin-dependent release of E4ICD and its recruitment to specific promoters, thereby regulating its transcriptional activity (see also Figure S5).

ErbB4 Is Necessary for the Correct Timing of Astrogenesis In Vivo

The in vitro studies suggested that presenilin-dependent ErbB4/TAB2/N-CoR nuclear signaling may regulate astrogenesis in vivo. To test this possibility, we analyzed the expression of the astrocytic markers GFAP and *S100* β in the cortex of *ErbB4* knockout embryos using a line of mutant mice that is rescued from early embryonic lethality by reintroduction of ErbB4 in the heart (Tidcombe et al., 2003). At E17.5, a time at which astrogenesis is just beginning, the levels of GFAP mRNA and protein were dramatically elevated in the cortical neurogenic layers of the *ErbB4*^{-/-} *HER4*^{heart} mice compared to their control littermates (*ErbB4*^{+/-} *HER4*^{heart}; Figures 6A and 6B). Furthermore, expression of *S100* β was also increased in the cortex of *ErbB4*^{-/-} *HER4*^{heart} mice (Figure 6C).

The results described above clearly indicated that ErbB4 signaling plays a critical role in controlling the onset of astrogenesis in vivo but did not provide insights into the importance of ErbB4 cleavage in this process. To investigate this, we tested whether the alterations in GFAP expression in the *ErbB4*^{-/-} mice could be rescued by re-expression of the different ErbB4 isoforms using in utero electroporation. cDNAs encoding either HER4 JMa or HER4 JMb were transfected into the cortices of E13.5 *ErbB4*^{-/-} *HER4*^{heart} mice together with a GFP expression plasmid (Figure S6A). When embryos were dissected immediately after transfection and slices of the forebrains incubated for 4 days, expression of cleavage-sensitive HER4 JMa, but not cleavage-resistant HER4 JMb, significantly reduced the GFAP expression levels (Figures 6D and 6E). Identical results were obtained when the embryos were allowed to develop in utero until E17.5 and then analyzed histologically (Figures 6F and 6G). These results indicate that canonical RTK signaling by ErbB4 is not sufficient for normal control of GFAP expression in the developing cortex and that ErbB4 cleavage is necessary to prevent precocious astrogenesis. Finally, to determine whether ErbB4 nuclear signaling is sufficient to repress

Expression of cleavage-sensitive HER4 JMa (arrow), but not cleavage-resistant HER4 JMb, significantly reduced the GFAP expression levels (red). Bar = 100 μ m.

(E) Quantitative analysis of GFAP expression in HER4 electroporated *ErbB4*^{-/-} *HER4*^{heart} brains sliced and cultured for 4 days in vitro (n = 3, *p < 0.05).

(F) Only HER4 JMa rescues the *ErbB4*^{-/-} phenotype in vivo. Day 13.5 *ErbB4*^{-/-} *HER4*^{heart} embryos were electroporated in utero with HER4 and GFP plasmids and allowed to develop until E17.5, when they were processed for GFAP immunofluorescence. Cortical coronal sections show that expression of cleavage-sensitive HER4 JMa (arrow), but not cleavage-resistant HER4 JMb, significantly reduced the GFAP expression levels (red). Bar = 50 μ m.

(G) Quantitative analysis of GFAP expression in HER4 electroporated *ErbB4*^{-/-} *HER4*^{heart} embryos (n = 3, *p < 0.05).

(H) Expression of activated E4ICD is sufficient to inhibit astrogenesis in vivo. Day 13.5 wild-type embryos were electroporated in utero with LexA-E4ICD constructs and GFP plasmids, allowed to develop until E18.5, and then analyzed for GFAP expression. Cortical coronal sections show that expression of WT LexA-E4ICD (arrow), but not KD LexA-E4ICD, significantly reduced the GFAP expression levels (red). Bar = 50 μ m.

(I) Quantitative analysis of GFAP expression in LexA-E4ICD electroporated wild-type embryos (n = 4, *p < 0.05).

astrogenesis in vivo, we tested whether expression of E4ICD would reduce GFAP expression in wild-type mice. Plasmids encoding either active or kinase-dead LexA-E4ICD (Figure S6B) were transfected into the brains of E13.5 wild-type embryos together with a GFP expression plasmid. After electroporation, the embryos were allowed to continue developing in utero until E18.5. Similar to what we observed in vitro (Figure 5D), expression of wild-type LexA-E4ICD significantly reduced the levels of GFAP compared to untransfected areas, whereas kinase-dead LexA-E4ICD did not (Figures 6H and 6I). These results show that E4ICD is necessary and sufficient to regulate astrogenesis in the intact developing cortex.

DISCUSSION

We have described a novel mechanism by which an RTK signals directly to the nucleus to regulate transcription and influence cell fate choices of NPs (Figure 7). During embryonic development, cortical NPs first generate neurons and then produce, or themselves become, astrocytes (Savaugeot and Stiles, 2002). However, the signaling mechanisms regulating the timing of these fate choices are not well defined. Particularly, it is unclear whether astrogenesis occurs later than neurogenesis because factors that induce astrocyte formation are produced after those inducing neurogenesis or because factors that inhibit astrogenesis are present at early stages of brain formation. Our results support the latter possibility, indicating that, during the early stages of brain development, NPs are exposed simultaneously to extracellular signals that induce neuronal and astrocyte production but that neurogenesis is favored by presenilin-dependent ErbB4 nuclear signaling that antagonizes the actions of astrogenesis-promoting signals. At later stages, reduction in ErbB4 signaling, most likely due to reduction in the levels of ErbB4 expression by the NPs (Fox and Kornblum, 2005), would favor the generation of astrocytes. It is likely that alterations in the timing of astrogenesis have effects on other aspects of brain development in mice with defective ErbB4 signaling. For example, it has been shown that *ErbB4* knockout mice have defects in tangential neuronal migration (Anton et al., 2004; Flames et al., 2004). Interestingly, we have shown that this type of migration is modulated by soluble signals produced by astrocytes (Mason et al., 2001). Thus, some of the defects in migration could be secondary to alterations in astrocyte number or function.

Our results reveal a role for presenilin in neural development, i.e., inhibition of astrogenesis via E4ICD release. Based on these findings, we considered the possibility that GFAP expression would be upregulated in *presenilin* $1^{-/-}$ mice as was the case for the *ErbB4* $^{-/-}$ mice. We used semiquantitative western blot analysis to measure the levels of GFAP protein in the brains of E17.5 embryos. Since *presenilin* $1^{-/-}$ mice have been shown to have a smaller number of NPs (Wines-Samuelson and Shen, 2005), we normalized the levels of GFAP to those of the NP marker nestin. Surprisingly, we found no differences

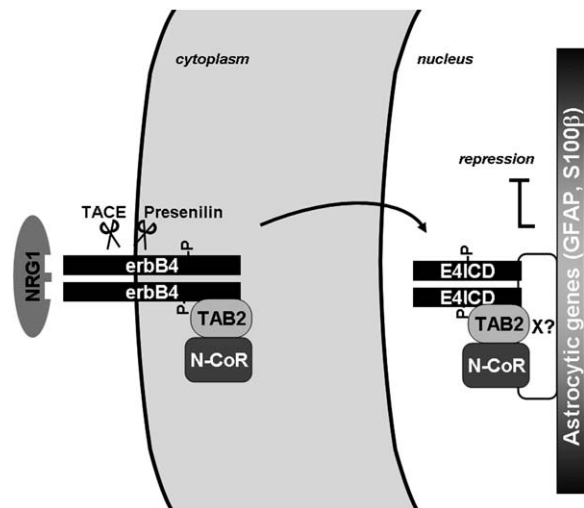


Figure 7. Mechanism for the Regulation of NP Fate by ErbB4 Nuclear Signaling

ErbB4 activation by NRG1 promotes receptor phosphorylation and its association with TAB2 and N-CoR. Ligand-induced ErbB4 cleavage by TACE and presenilin allows for the nuclear translocation of the E4ICD/TAB2/N-CoR complex. This complex binds to specific promoters (GFAP and *S100β*), leading to their transcriptional repression and preventing their activation by factors that induce astrocyte differentiation, such as CNTF.

in GFAP expression between *presenilin* $1^{-/-}$ mice and their control littermates (S.P.S. and G.C., unpublished data). Presenilin also plays important roles in Notch cleavage and function, and Notch1 signaling promotes astrogenesis (Gaiano and Fishell, 2002). It is possible that the combined loss of an inducer of astrogenesis (Notch) and an inhibitor of astrogenesis (ErbB4 nuclear signaling) results in an apparently normal level of GFAP expression in *presenilin* $1^{-/-}$ mice, but this merits further investigation. Nevertheless, these observations, together with our in vitro studies, underscore the high degree of integration necessary for NPs to interpret multiple signaling pathways, such as ErbB, Notch, and cytokine receptors, in order to make adequate and timely fate choices.

We have also uncovered a role for presenilin-dependent proteolytic cleavage of transmembrane proteins in cell signaling, i.e., presenilin activity is required for transcriptional repression and cell fate specification through cleavage of an RTK. Alterations in presenilin/ γ -secretase activity are associated with Alzheimer's disease (AD) (Hutton and Hardy, 1997), and ErbB4 is found at high levels surrounding neuritic plaques in AD brains (Chaudhury et al., 2003). Together, these observations suggest that changes in presenilin-dependent ErbB4 signaling could be involved in AD. One way in which this could occur is through cross-talk between ErbB4 and the amyloid precursor protein (APP). Presenilin-dependent cleavage of APP results in the release of its intracellular domain (AICD), which has been suggested to function in nuclear signaling and

interacts with TAB2 and N-CoR. In transfected cells, AICD has been shown to displace N-CoR from specific promoters, resulting in gene derepression (Baek et al., 2002). Thus, alterations in the balance between AICD and E4ICD due to defects in APP and ErbB4 processing could affect the transcriptional regulation of critical genes and contribute to neurodegeneration. Furthermore, recent work has demonstrated that complete loss of presenilin activity in the forebrain leads to memory deficits, synaptic dysfunction, and neurodegeneration without generation of amyloid plaques (Saura et al., 2004). These results suggest that some of the neurodegenerative processes that might contribute to the severity of AD could be induced independently from abnormal amyloid peptide accumulation. In this regard, ErbB4 signaling regulates several aspects of neuronal development and function beyond neurogenesis, including dendrite morphology (Rieff and Corfas, 2006), neurotransmitter receptor expression (Ozaki et al., 1997; Rieff et al., 1999; Liu et al., 2001; Okada and Corfas, 2004; Gu et al., 2005), and neuronal survival (Li et al., 2003), all of which have been implicated in AD pathology. Another observation suggesting that defects in ErbB4 signaling could be involved in AD is that GFAP and S100 β expression are increased in AD (Beach et al., 1989; Mrak and Griffin, 2001). Interestingly, S100 β overexpression has been associated with increased susceptibility to β -amyloid toxicity (Craft et al., 2005). Therefore, defects in presenilin-dependent ErbB4 signaling could lead to increased S100 β expression, which in turn could contribute to AD by potentiating the degenerative process initiated by β -amyloid. Finally, an intriguing possibility is that ErbB4 nuclear signaling could also regulate the differentiation of adult NPs and that defects in this pathway might result in alterations in adult neurogenesis, which may also contribute to AD (Feng et al., 2001).

In summary, this study uncovers a signaling mechanism for an RTK, demonstrates roles for presenilin in the developing nervous system, and provides insights into the mechanisms that control the timing of astrogenesis in the developing brain. Together, our results indicate that further studies of ErbB4 nuclear signaling could provide important insights into the mechanisms of brain development and the causes of neurodegeneration. Furthermore, our findings on ErbB4 nuclear signaling might be relevant to other RTKs since other receptors undergo ligand-induced shedding of their extracellular domain like ErbB4, and this type of shedding has been shown to trigger presenilin-dependent cleavage of numerous proteins (Carpenter, 2003).

EXPERIMENTAL PROCEDURES

Antibodies

Antibodies used were mouse FLAG M2 and β -tubulin III (Sigma), GFAP and nestin (Chemicon), and 4G10 (gift from O. Gjoerup and T. Roberts, Dana-Farber Cancer Institute); rabbit TAB2 (ABR), ErbB4 (Santa Cruz), N-CoR (Upstate), GFAP (DAKO), and LexA (Abcam); and goat TAB2 and S100 β (Santa Cruz).

Yeast Two-Hybrid Screen

Yeast two-hybrid screen was performed as described in Finley and Brent (1996) (see Supplemental Experimental Procedures) with a bait containing the entire E4ICD in an activated state and a cDNA expression library from rat E14 spinal cord and dorsal root ganglia.

Plasmids

Full-length cDNAs encoding human ErbB4 (HER4 JMa and JMb) were cloned into pCDNA3. Kinase-dead HER JMa^{KD} (K751M) and presenilin-resistant HER4 V673I (Vidal et al., 2005) were generated by site-directed mutagenesis. Wild-type and kinase-dead LexA-E4ICD were cloned into pCDNA3 for expression in mammalian cells. The expression vector for FLAG-TAB2 was generated by cloning mouse TAB2 into pA1fg (gift from M. Lin and M. Greenberg, Children's Hospital Boston). FLAG-TAB2 Δ C (amino acids 1–628) and TAB2 Δ N (amino acids 629–693) were generated by PCR and cloned into pA1fg. Wild-type and dominant-negative presenilin plasmids (pZeo-PS1, pZeo-PS1 D257A, and pZeo-PS1-D385A) were a gift from D. Selkoe.

Cell Lines and Transfection

NIH 3T3 cells stably expressing HER4 JMa or JMb have been described previously (Rio et al., 2000). HEK293 and NIH 3T3 cells were cultured in DMEM (Invitrogen) containing 10% HI-FBS and 1% penicillin/streptomycin. For transient transfections, cells were plated at 70%–80% confluency on six-well or 10 cm plates, transfected using FuGENE 6 (Roche), and harvested 24 hr later. When indicated, cells were treated with recombinant human neuregulin 1- β 1 (NRG1, 1 nM, R&D Systems). DAPT (1 μ M, Calbiochem) was added 30 min before NRG1.

Coimmunoprecipitation and Western Blot

Cells were washed with cold PBS on ice and lysed in 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate, 1 mM Na₃VO₄, and 1 mM Pefabloc (Roche Applied Science). For immunoprecipitations, lysates were precleared with agarose-conjugated normal IgG for 30 min and immunoprecipitated with agarose-conjugated ErbB4 antibody (Santa Cruz) or FLAG antibody (M2, Sigma) overnight at 4°C. Samples were washed four times with lysis buffer before the beads were resuspended in SDS sample buffer and boiled for 2 min. Samples were subjected to SDS-PAGE. Western blotting was performed by standard protocols and developed using ECL reagents (Amersham).

Primary Neural Precursor Cultures

Timed-pregnant Long-Evans rats (Charles River Laboratories) were treated according to the guidelines of the Animal Care and Use Committee of Children's Hospital Boston. NPs were prepared from E14.5 rat telencephalons and cultured as described (Bonni et al., 1997). For differentiation assays, cells were treated with CNTF (0.3 ng/ml, Upstate) or PDGF (10 ng/ml, PeproTech).

RT-PCR Analysis of ErbB Receptors

After reverse transcription of total RNA from NPs, cDNA was amplified by PCR using intron-spanning primers that differentiate between the two ErbB4 juxtamembrane isoforms (Elenius et al., 1997). Primers sequences are provided in Supplemental Experimental Procedures.

Preparation of Cytoplasmic Fractions

Cytoplasmic fractions of NPs were prepared as described (Vidal et al., 2005).

Immunofluorescence Analyses of Cells in Culture

Cells grown on glass coverslips were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized in 0.1% Triton X-100 in PBS for 10 min, blocked with 5% BSA in PBS for 30 min, and then probed overnight at 4°C with primary antibodies diluted in blocking solution. The coverslips were washed with PBS, and detection was performed using

the appropriate fluorescent secondary antibodies (Jackson Immuno-Research) for 1 hr at room temperature. Nuclei were counterstained with Hoechst 33342 (1 nM, Invitrogen). Coverslips were washed three times for 10 min in PBS and mounted with VECTASHIELD (Vector Laboratories). Images were obtained using a Zeiss LSM 510 confocal microscope and taken with the same exposure parameters in each experiment.

Luciferase Reporter Assays

Mouse TAB2 was cloned into pM plasmid (Invitrogen) to generate the GAL4-TAB2 fusion protein. The *MH100-TK*-luciferase reporter construct, containing five copies of the *GAL4* DNA binding site and the *TK* promoter upstream of a luciferase reporter gene, was provided by R. Evans (Salk Institute). *GFAP*-luciferase and *S100 β* -luciferase reporters were provided by M. Greenberg and B. Yankner (Children's Hospital Boston), respectively. The dual luciferase assay (Promega) was performed according to the company's recommendations.

Generation of Lentivirus for shRNA Expression

The vector pLL3.7 (from L. Van Parijs) was used to generate shRNAs. The nucleotide sequences for the shRNAs are provided in [Supplemental Experimental Procedures](#). Lentiviral production was performed as described (Rubinson et al., 2003).

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation was performed on NPs using the ChIP Assay Kit (Upstate) according to supplier's recommendations. PCR was performed at 38 cycles as described (Hermanson et al., 2002). (For primers, see [Supplemental Experimental Procedures](#).)

ErbB4 Knockout Mice

We used *ErbB4*^{-/-} mice that were rescued from embryonic lethality by cardiac expression of HER4 (Tidcombe et al., 2003). *ErbB4*^{-/-} *HER4*^{heart} males were crossed with *ErbB4*^{+/-} *HER4*^{heart} females to generate *ErbB4*^{-/-} (null) and *ErbB4*^{+/-} (control) *HER4*^{heart} embryos. The use of the animals was approved by the Animal Care and Use Committee of Children's Hospital Boston.

In Situ Hybridization

In situ hybridization and preparation of digoxigenin-labeled riboprobes were performed as previously described (Redwine and Armstrong, 1998). Riboprobes were generated using a *GFAP* 1.1 kb fragment (gift from R. Skoff, Wayne State University).

In Utero Electroporation and Slice Cultures

Detailed methods for in utero electroporation and slice culture are available in [Supplemental Experimental Procedures](#).

Immunohistochemistry

Tissue sections were blocked with 0.1% Triton X-100, 5% BSA in PBS for 30 min and stained overnight with GFAP (DAKO) or S100 β antibody (Santa Cruz). Primary antibody was then visualized using a Cy3- or Cy5-conjugated secondary antibody (Jackson ImmunoResearch). Nuclei were counterstained with Hoechst 33342 (1 nM, Invitrogen). Fluorescent images were captured with a Nikon microscope (Eclipse E800) using Spot advanced image software (version 3.4.4) or with a Zeiss LSM 510 confocal microscope for quantitative analysis.

Quantification of GFAP Immunofluorescence

Confocal images of GFAP immunostaining in the areas adjacent to the lateral ventricles were taken with the same exposure parameters for each experiment. The same areas were imaged for GFP fluorescence. The mean intensity of GFAP immunofluorescence in the transfected (GFP+) and untransfected (GFP-) regions was then calculated using Photoshop CS (Adobe). The ratio of GFAP intensity (GFP+/GFP-) was calculated for each section. At least three independent embryos were analyzed for each experimental condition.

Statistical Analysis

All data is presented as mean \pm SEM. Statistical significance for neuronal and astroglial differentiation assays was determined by χ^2 . More than 300 cells were analyzed for every condition in each of three independent experiments. Statistical significance for nuclear translocation of E41CD, TAB2, and N-CoR was determined by χ^2 , with more than 100 cells analyzed per experimental condition. Statistical significance for GFAP quantitative analysis was determined by Student's t test.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and six figures and can be found with this article online at <http://www.cell.com/cgi/content/full/127/1/185/DC1/>.

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